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14. ABSTRACT

The overall goal of this project is to understand how β_2AR signaling in macrophages contributes to Acute Respiratory Distress Syndrome, which is a significant contributor to morbidity and mortality in military and civilian settings. To achieve this goal, we proposed three specific aims. In Aim 1, we proposed to determine whether β_2-agonists worsen influenza A-induced lung injury via β_2ARs on tissue-resident and/or monocyte-derived alveolar macrophages. In Aim 2, we aimed to investigate whether IL-6 and/or recruitment of monocyte-derived macrophages are required for the effects of β_2ARs activation on influenza A-induced lung injury. In Aim 3, we proposed to determine whether inhibition of β_2ARs attenuates age-related worsening of influenza A-induced acute lung injury. In this reporting period, we confirmed that β_2ARs on monocytes and macrophages are critical for influenza A virus-induced acute lung injury. We developed and validated a method to clearly identify tissue resident and monocyte-derived macrophages. Our findings suggest that recruited monocyte-derived, but not resident macrophages are responsible for the influenza-induced acute lung injury. We also discovered that β_2AR signaling in macrophages may regulate influenza-induced metabolic changes, which are required for pro-inflammatory response against influenza suggested by attenuation of inflammation with inhibitors of glycolysis (Hk2) and carbonic anhydrase (Ca2).

15. SUBJECT TERMS
Acute lung injury, Acute Respiratory Distress Syndrome, ARDS, pulmonary edema, influenza, viral pneumonia, inflammation, macrophage, transcriptome, RNA sequencing, metabolism, catecholamine, albuterol, epinephrine.

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1. INTRODUCTION

The overall goal of this project is to understand how β_2AR signaling in macrophages contributes to Acute Respiratory Distress Syndrome (ARDS). ARDS is a significant contributor to morbidity and mortality in military and civilian settings resulting in 75,000 deaths annually in the U.S. The treatment of ARDS is supportive. Despite improvement in supportive care, the mortality from ARDS remains as high as 45%. A common asthma medication, β_2-agonists have been suggested as a promising therapy for ARDS due to their ability to improve lung edema clearance via β_2-adrenergic receptors (β_2ARs) on alveolar epithelial cells. However, recent clinical trials in ARDS have reported unexpected harm with the beta2-agonists. Our preliminary data suggested that activation of β_2ARs on alveolar macrophages may be responsible for the unexpected harm with the beta2-agonists. Based on these data, we hypothesized that activation of β_2ARs promotes ARDS via an exaggerated IL-6 response and inhibition of the recruitment of monocyte-derived alveolar macrophages. We will test our hypothesis in three specific aims using influenza as a model of ARDS. In Aim 1, we will determine whether β_2-agonists worsen influenza A-induced lung injury via β_2ARs on tissue-resident and/or monocyte-derived alveolar macrophages. In Aim 2, we will investigate whether IL-6 and/or recruitment of monocyte-derived macrophages are required for the effects of β_2ARs activation on influenza A-induced lung injury. In Aim 3, we will determine whether inhibition of β_2ARs attenuates age-related worsening of influenza A-induced acute lung injury.

2. KEYWORDS

Acute lung injury, Acute Respiratory Distress Syndrome, ARDS, pulmonary edema, influenza, viral pneumonia, inflammation, macrophage, transcriptome, RNA sequencing, metabolism, catecholamine, albuterol, epinephrine.

3. ACCOMPLISHMENTS

3a. What were the major goals of the project?

The overarching goal of this project is to determine how β_2AR signaling in macrophages contributes to the development of ARDS. In the SOW, we stated two major tasks under Specific Aim 1 within the first 12 months. Major task 1 was to generate shielded and unshielded bone marrow chimeric mice. Major task 2 was to determine the effect of β_2AR activation/inhibition on tissue-resident and/or monocyte-derived alveolar macrophages during acute lung injury.

We completed all subtasks and milestones in major task 1. We also completed 75% of all subtasks and milestones in major task 2. We anticipate that we will complete this subtask 2 including the analysis of RNA sequencing data in the next 3 months.

Specific Aim 1: Determine whether β_2-agonists worsen influenza A-induced lung injury via β_2ARs on tissue-resident and/or monocyte-derived alveolar macrophages.	**Timeline**	**Percent Completion**
Major Task 1: Generation of shielded and unshielded bone marrow chimeric mice	Months	
Subtask 1: Optimize of the bone marrow chimera generation	1-2	100%
Subtask 2: Optimize flow cytometry techniques to sort lung macrophage populations	1-2	100%
Milestone(s) Achieved: Optimization of the bone marrow chimera generation and flow cytometry to identify different populations of lung macrophages.	3	100%

Local IACUC Approval	1	100%
Milestone(s) Achieved: ORP/ACURO Approval	1-3	100%
Major Task 2: Determine the effect of β_2AR activation/inhibition on tissue-resident and/or monocyte-derived alveolar macrophages during acute lung injury.		
Subtask 1: Induction of acute lung injury using intratracheal administration of influenza A virus and testing the effect of β_2AR inhibition and activation	4-12	75%
Subtask 2: Obtain high-quality RNA from macrophage populations for RNAsequencing.	4-12	75%
Milestone(s) Achieved: Characterize the role of β_2AR signaling on different populations of lung macrophages during acute lung injury	12	75%
Specific Aim 2: Determine whether IL-6 and/or recruitment of monocyte-derived macrophages are required for the effects of β_2ARs on influenza A-induced lung injury.		
Major Task 3: Determine the role of IL-6 and recruited monocytes in the pathogenesis of acute lung injury		
Subtask 1: Complete proposed studies in IL-6 knockout mice.	12-15	10%
Subtask 2: Perform proposed experiments using anti-CCR2 antibody.	15-17	0%
Subtask 3: Cross and breed CXCR1^{CreER}/β_2AR$^{-/-}$ mice and perform proposed experiments in these mice.	12-21	30%
Subtask 4: Perform RNA sequencing and data analysis from experiments proposed in Aims 1 and 2.	21-24	0%
Milestone(s) Achieved: Determine whether IL-6 and/or monocyte-derived macrophages are required for acute lung injury	24	0%
Specific Aim 3: Determine whether inhibition of β2ARs attenuates age-related worsening of influenza A-induced lung injury.		
Major Task 4: Generation of aged mice and completion of experiments proposed in aged mice		
Subtask 1: Generate aged mice in sufficient numbers to perform experiments	1-27 (will start on day 1)	20%
Subtask 2: Perform experiments proposed in Aim 3.	24-36	0%
Subtask 3: Perform RNA sequencing and data analysis from experiments proposed in Aim 3.	24	0%

Milestone(s) Achieved: Determine whether age-related elevation in catecholamines are responsible for age-related increased mortality from influenza A-induced ARDS.	36	0%

3b. What was accomplished under these goals?

3b1. Major activities

The specific aims of the project have not been modified. The primary goal of the project remains understanding how β_2AR signaling in macrophages contributes to the development of ARDS. Specifically, we will investigate the role of endogenous (catecholamines, which are elevated during critical illness and ARDS) and exogenous (β_2-agonists, which are the most commonly prescribed asthma medications) affect lung macrophage function. We will also investigate the role of tissue resident and bone marrow derived macrophages and delineate how β_2AR signaling in these different lung macrophage populations.

We completed the experiments and sample collection in Aim 1.1, which aimed to determine whether β2-agonist therapy worsens influenza A-induced lung injury via β2-adrenergic receptors on macrophages and monocytes. We have also completed the experiments and sample collection from tissue resident and monocyte-derived macrophages as we proposed in Aim 1.2. We isolated the RNA from these samples. RNA integrity number (RIN) has been >9 confirming high quality RNA. The sequencing of these RNA samples will be performed when all samples in Aim 1 are collected.

Approaches taken to ensure robust and unbiased results:
-We optimized and validated the flow cytometry techniques to sort lung macrophage populations.
-We confirmed that $\beta_2AR^{-/-}$ are genetically C57BL/6 background.
-We validated the bone marrow chimera generation methodology.
-The titration of influenza A virus was standardized with the assistance of Dr. Balaji Manicassamy who is an expert in influenza in the department of microbiology at the University of Chicago.
-All tissues are collected before RNA is isolated and sequencing is performed to minimize potential "batch" effect.
-The samples are deidentified so that bioinformaticians who will analyze the data are blinded to the treatments each sample has been subjected to.

3b2. Specific objectives

The specific objectives and aims of the project did not change. Despite an unexpected delay due to the background genotype and breeding of mice, we have made significant progress to complete tasks and meet milestones as we proposed in our project as summarized in 3a. The progress that we have made in specific objectives so far is described below.

Optimization of bone marrow chimeras and flow cytometric analysis of lung immune cells. This task was completed in the first 3 months. The methodology for flow cytometry and bone marrow chimera generation are published in Misharin *AJRCMB* 2013 and Misharin *JEM* 2017, respectively.

Establishment of PR8 influenza A virus-induced acute lung injury. As recommended by the research community, we also established the acute lung injury model using a different mouse-adapted influenza A virus (PR8). While WSN and PR8 are similar strains, PR8 is the most commonly used strain in mouse models. We also wanted to confirm that our findings using WSN are not strain specific. This task was completed within the first 3 months. During titration experiments, we found that 100 pfu of PR8 virus induced severe acute lung injury similar to WSN. Acute lung injury was detectable on day 3 and peaked on day 6. PR8 caused significant morbidity assessed by weight and muscle loss, which also became clinically apparent on day 6. Therefore, we focused our analysis on days 3 and 6.

Problems encountered: We had an unexpected delay in starting the chimera experiments because initial experiments using our $\beta_2AR^{-/-}$ and C57BL/6 ($\beta_2AR^{+/+}$) mice led to immune response consistent with rejection. We determined that our $\beta_2AR^{-/-}$ mice was not fully backcrossed to C57BL/6. With the help of speed congenic system, we fully-backcrossed $\beta_2AR^{-/-}$ mice to C57BL/6 after two additional crosses. However, this process led to an approximately 6 months of delay in starting the chimera experiments.

Aim 1. Determine β₂-agonists worsen influenza A-induced lung injury via β₂ARs on tissue-resident and/or monocyte-derived alveolar macrophages. In Aim 1, our goal was to confirm the role of β_2AR signaling in macrophages/monocytes in acute lung injury as our preliminary data in mice lacking β_2ARs in alveolar macrophages (*LysM-Cre;*$\beta_2AR^{fl/fl}$), or alveolar epithelial cells (*SPC-Cre;*$\beta_2AR^{fl/fl}$) showed that loss of β_2ARs in macrophages is associated with improved survival from acute lung injury.

Aim 1.1. To confirm our preliminary data from *LysM-Cre;*$\beta_2AR^{fl/fl}$ and *SPC-Cre;*$\beta_2AR^{fl/fl}$ mice, we generated unshielded bone marrow chimeras using $\beta_2AR^{+/+}$ and $\beta_2AR^{-/-}$ mice. Similar to our results in *LysM-Cre;*$\beta_2AR^{fl/fl}$ mice, transplantation of bone marrow from $\beta_2AR^{-/-}$ (donor) mice into $\beta_2AR^{+/+}$ (recipient) mice resulted in attenuation of influenza A-induced acute lung injury (Figure 1). Similar response was seen in $\beta_2AR^{-/-}$ (donor)/$\beta_2AR^{-/-}$ (recipient) chimeras. Transplantation of bone marrow from $\beta_2AR^{+/+}$ (donor) mice into $\beta_2AR^{-/-}$ or $\beta_2AR^{+/+}$ (recipient) mice did not alter the severity of acute lung injury. Consistent with the attenuation of acute lung injury, *il6* mRNA in macrophages and IL-6 protein in bronchoalveolar lavage (BALF) was lower in $\beta_2AR^{-/-}$ (donor)/$\beta_2AR^{-/-}$ (recipient) and $\beta_2AR^{+/+}$ (donor)/$\beta_2AR^{-/-}$ (recipient). In contrast to our preliminary data showing increased recruitment of macrophages, analysis of cell count and differential in bone marrow chimeric mice from $\beta_2AR^{-/-}$ (donor) mice was not different (data not shown). We completed all of experiments we proposed in Aim 1.1.

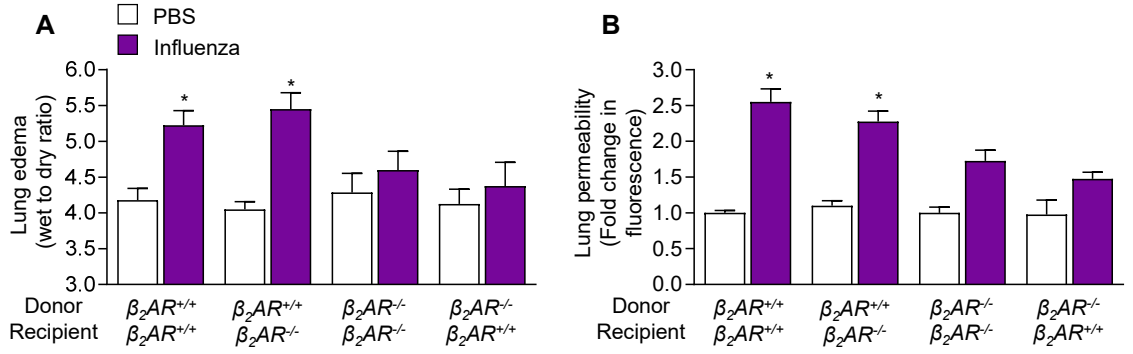

Figure 1. Loss of β₂AR on monocytes/macrophages attenuate influenza A virus-induced acute lung injury. We generated unshielded bone marrow chimeric mice using $\beta_2AR^{+/+}$ and $\beta_2AR^{-/-}$ mice. Donor and recipient strains are as indicated. Once chimera is confirmed, mice were infected with influenza A virus (PR8, 100 pfu, intratracheally). Six days later, we measured **(A)** lung edema using wet-to-dry ratio and **(B)** lung permeability using 4kD dextran. While transplantation of bone marrow from $\beta_2AR^{+/+}$ (donor) did not alter the severity of acute lung injury, transplantation of bone marrow from $\beta_2AR^{-/-}$ attenuated the severity of acute lung injury.

We also treated a group of chimeric mice with formoterol, a long acting β_2-agonist as described in our proposal. Chimeric mice with monocytes/macrophages expressing *β_2ARs* showed worsening of the severity of acute lung injury (Figure 2). In contrast chimeric mice with monocytes/macrophages with the loss of *β_2ARs* did not respond to formoterol. Collectively, these data confirm our preliminary findings that β_2AR signaling on macrophages regulates the severity of acute lung injury; inhibition of β_2ARs attenuate acute lung injury, whereas their activation worsen acute lung injury.

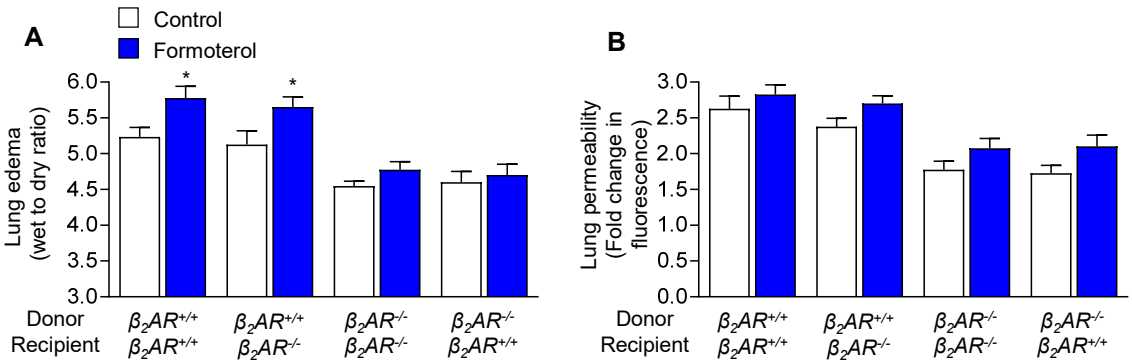

Figure 2. Activation of β$_2$ARs on monocytes/macrophages worsen the severity of influenza A virus-induced acute lung injury. We generated unshielded bone marrow chimeric mice using $\beta_2AR^{+/+}$ and $\beta_2AR^{-/-}$ mice. Donor and recipient strains are as indicated. Once chimera is confirmed, mice were infected with influenza A virus (PR8, 100 pfu, intratracheally). Mice also received either formoterol, a long-acting β$_2$-agonist or control vehicle. Six days later, we measured **(A)** lung edema using wet-to-dry ratio and **(B)** lung permeability using 4kD dextran. The effect of formoterol was only seen in mice with monocytes/macrophages expressing $\beta_2AR^{+/+}$ (Donor: $\beta_2AR^{+/+}$). Loss of β_2AR prevented the effects of formoterol on acute lung injury.

To understand the transcriptomic changes in alveolar macrophages induced by influenza A virus, we infected C57BL/6 mice with influenza A virus. Six days after infection, we collected alveolar macrophages for RNA sequencing. Transcriptomic data showed upregulation of interferon target genes and antiviral genes (*Oas, Mx1*) (Figure 3). In addition to antiviral genes, influenza induced metabolic genes (*Irg1, Ca2, Pfkfb3, Hk2*), which were among top 100 most upregulated genes. These results suggest that influenza infection induces metabolic reprogramming similar to LPS as recently reported.

We confirmed these metabolic changes in vitro. Macrophages infected with influenza virus showed increased expression of glycolytic metabolic genes and Irg1 and exhibited increased glycolysis (measured by Seahorse XF24). Importantly, inhibition of glycolysis or carbonic anhydrase attenuated influenza induced IL-6 response from macrophages. These results suggested influenza induced metabolic changes in macrophages drive immune response against influenza virus.

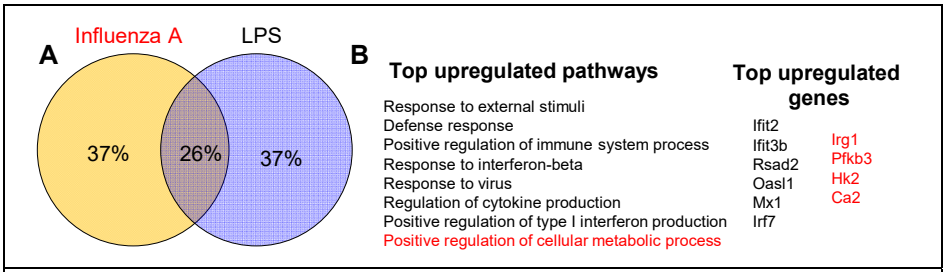

Figure 3. Transcriptomic response in alveolar macrophages after influenza A infection. C57BL/6 mice were infected with PR8 influenza A virus (100 pfu intratracheally). Alveolar macrophages were isolated for RNA sequencing on day 6. **(A)** Venn diagram shows the differences and similarities in transcriptomic data after influenza and LPS treatment. **(B)** Top upregulated pathways identified by IPA and **(C)** top upregulated genes after influenza A virus are shown. Cellular metabolic process and metabolic genes (shown in red font) were upregulated by influenza A virus. Irg1; Immune response gene 1, Pfkfb3; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, Hk2: hexokinase 2, Ca2; carbonic anhydrase 2.

Interestingly, influenza-induced increase in the expression of these metabolic genes was inhibited in C57BL/6 mice treated with propranolol and $\beta_2AR^{-/-}$ mice. In contrast, formoterol treatment further increased the expression of these metabolic enzymes. Promoter regions of Pfkfb3, and Ca2 genes possess CREB transcription factor binding and thus can be affected by β_2AR activation. These results suggest that inhibition of β_2AR signaling prevents influenza-induced metabolic reprogramming, which drive inflammatory response from alveolar macrophages.

Aim 1.2. We proposed to determine the relative contribution of tissue resident alveolar macrophages (TR-AMs) and recruited monocyte (Mo)-derived alveolar macrophages (Mo-AMs) to acute lung injury and investigate the role of β_2AR signaling in different macrophage populations.

In order to achieve this goal, we proposed to do lung-shielded bone chimera method as this approach would allow us to determine the specific role of β_2ARs on Mo-AMs and distinguish their response from that of the TR-AMs. We have recently published the methodology on thoracic/lung shielding that we described in our project (Misharin AV, et al. *J Exp Med* 2017). This manuscript was reviewed by *Nature*, which declined its publication prior to getting it published in *JEM*. During its review in *Nature*, the reviewers raised concerns about the potential impact of radiation and myeloablative chemotherapy on immune response and the validity of our findings. We received similar criticism about the potential impact of bone marrow suppression when we presented our methodology at the American Thoracic Society conference last year.

To overcome these concerns, reviewers and our colleagues recommended the use of dye-based methodology. Although our results were confirmatory of our findings that we obtained with *LysM-Cre;$\beta_2AR^{fl/fl}$* and *SPC-Cre;$\beta_2AR^{fl/fl}$* mice, we decided to implement this method in our laboratory as recommended. In this method that allows us to specifically evaluate TR-AM and Mo-AM populations in the lung, mice are treated with PKH26 Red Fluorescent Cell Linker dye (Sigma) 1 day prior to influenza infection. The PKH26 labels the lipid membrane of resident cells, but not the bone marrow cells from which infiltrating macrophages (Mo-AMs) arise. Following influenza infection, alveolar macrophages are collected and stained with F4/80 antibody to select for macrophages. Then TR-AMs (PKH26+) and recruited Mo-AMs (PKH26-) are flow-sorted based on PKH26 fluorescence. This method has several advantages over the lung-shielded bone marrow chimera method. (1) It eliminates the need for radiation and myeloablative chemotherapy, which themselves may affect the results. (2) It decreases the number of mice needed for the experiments.

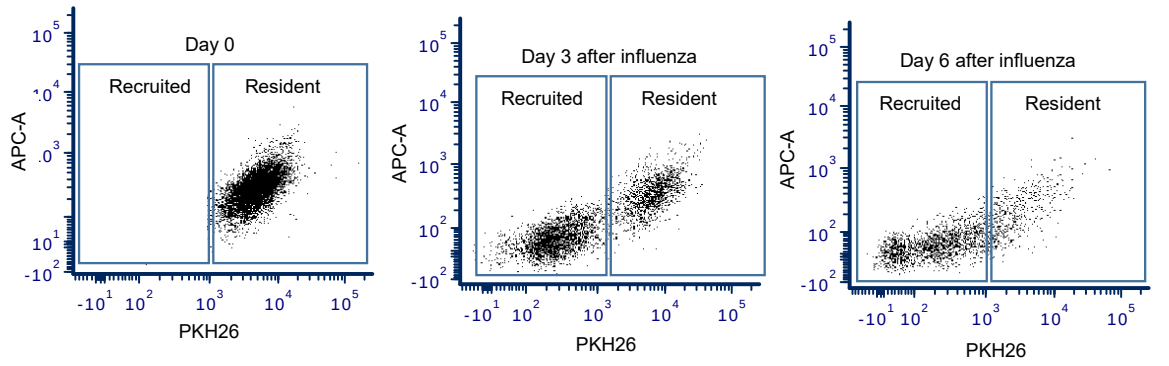

Figure 4. Identification of tissue resident (TR-AMs) and monocyte-derived (Mo-AMs) alveolar macrophages using PKH26 dye method. Mice are treated with PKH26 Red Fluorescent Cell Linker dye (Sigma) 1 day prior to influenza infection. The PKH26 labels the lipid membrane of resident cells, but not the bone marrow cells from which infiltrating macrophages (Mo-AMs) arise. Following influenza infection, cells were collected and stained with F4/80 antibody to select for macrophages. Then TR-AMs (PKH26+) and recruited Mo-AMs (PKH26-) were flow-sorted based on PKH26 fluorescence. Flow cytometry plots show that TR-AMs are the only subpopulation of AMs on day 0 and they decrease overtime. In contrast, Mo-AMs are not present on day 0 and significantly increase in number on day 3 after influenza A infection and become the major subpopulation of macrophage on day 6.

In preliminary experiments, we found that PKH26 dye method allows clear separation of the TR-AMs from Mo-AMs, which appeared in significant numbers in day 3 and further increased on day 6. Interestingly, TR-AMs decreased gradually from day 3 to day 6. (Figure 4) On day 6, majority of AMs in the lungs were recruited Mo-AMs. Assessment of il6 mRNA showed that influenza A virus induced a gradual increase in il6 mRNA in TR-AMs. The expression of IL6 and TNFα mRNA in Mo-AMs was significantly higher in Mo-AMs compared with that in TR-AMs suggesting that Mo-AMs are responsible for the macrophage-

driven inflammatory response against influenza A virus (Figure 5).

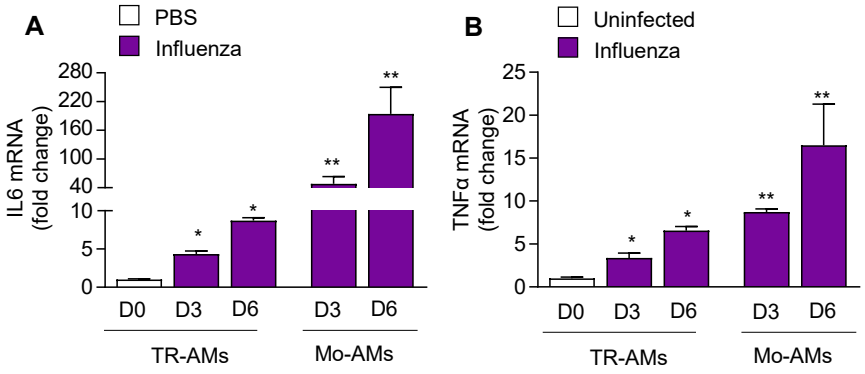

Figure 5. Influenza-induced pro-inflammatory cytokine expression is substantially higher in recruited monocyte-derived macrophages compared with tissue resident alveolar macrophages. We pretreated C57BL/6 mice with PKH26 before we infected them with PR8 influenza A virus (100 pfu intratracheally). Alveolar macrophages were isolated and flow-sorted based on PKH26 fluorescence to distinguish TR-AMs (PKH26+) from recruited Mo-AMs (PKH26-) on day 0 (uninfected), day 3 and day 6 after influenza infection. We then isolated qRT-PCR for **(A)** IL-6 and **(B)** TNFα mRNA expression.

In addition to assessment of inflammatory response, we also measured the mRNA expression of metabolic genes that we identified. Using qRT-PCR, we measured mRNA expression of Hk2, Pfkfb3, Ca2, and Irg1 in TR-AMs and Mo-AMs at different time points after influenza infection. Similar to what we observed in the expression of pro-inflammatory cytokines (Figure 5), the expression of these metabolic genes was time-dependent and higher in Mo-AMs compared to TR-AMs (Figure 6).

We expect to complete the experiments using the PKH26 dye method and the lung-shielded bone marrow chimera experiments in the next 3 months. Once all experiments are completed, we will isolate the RNA for sequencing to minimize the batch effect. The RNAseq and analysis should be completed within the next 3-4 weeks after collection process is completed.

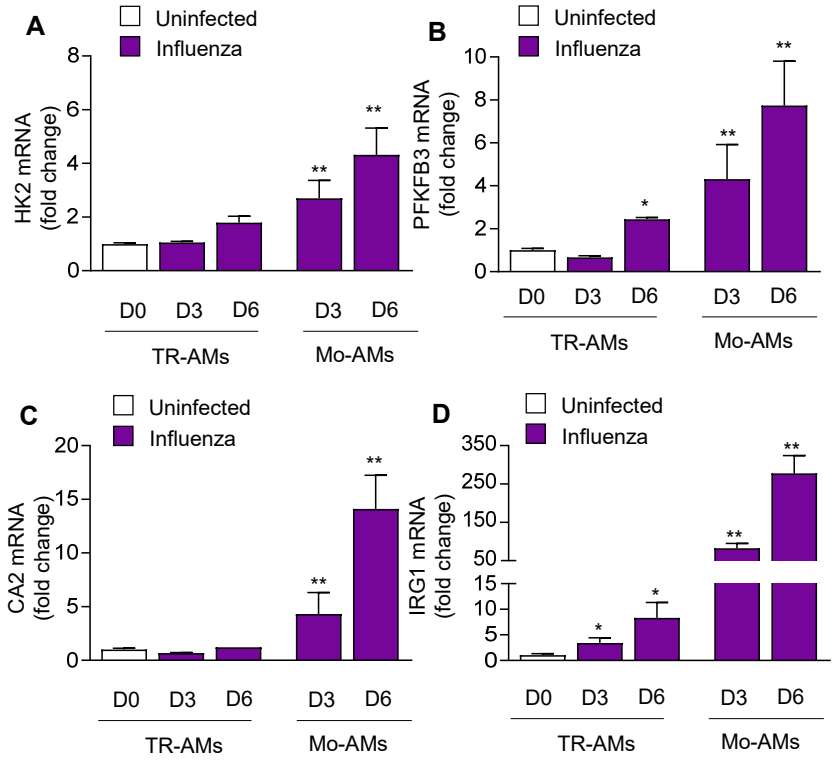

Figure 6. Influenza induces metabolic gene expression in recruited Mo-AMs compared with TR-AMs after influenza A infection. We measured mRNA expression of metabolic genes; **(A)** HK2, **(B)** PFKFB3, **(C)** CA2 and **(D)** IRG1 in TR-AMs and Mo-AMs isolated from mice at different times (day 0, 3 and 6) after influenza A infection.

Aim 2. Determine whether IL-6 and/or recruitment of monocyte-derived macrophages are required for the effects of β_2ARs on influenza A-induced lung injury. We are ready to start experiments that we proposed in Aim 2.2. We are currently crossing the *il6*$^{-/-}$ mice with *β_2AR*$^{-/-}$ mice. We anticipate to start the proposed experiments in Aim 2.1. in December 2017 and those in Aim 2.3. in 6 months.

Aim 3. Determine whether inhibition of β_2ARs attenuates age-related worsening of influenza A-induced lung injury. We have set aside both *β_2AR*$^{+/+}$ *and β_2AR*$^{-/-}$ mice to age in preparation to study the effect of aging on influenza A-induced lung injury and putative role of β_2AR signaling in macrophages in this process.

3b3. Significant results or key outcomes

1. Using unshielded bone marrow chimeras, we have confirmed that the β_2ARs on monocytes and macrophages are critical for influenza A virus-induced acute lung injury. Specifically, we found that inhibition of the effects of endogenous catecholamines via engagement of β_2ARs on macrophages/monocytes attenuate the severity of influenza A-induced lung injury, and associated cytokine (IL-6) release. Administration of β_2-agonists further worsen acute lung injury and IL-6 release.

2. We developed a method to successfully label TR-AMs using PKH26 to distinguish TR-AMs from Mo-AMs. This method allows us to study the two distinct macrophage populations without the need to use bone marrow chimerism eliminating the potential confounding effects of radiation and myeloablative chemotherapy on results.

3. Influenza virus induces a gradual reduction of TR-AMs and in contrast it causes an increase in Mo-AMs, which represent the majority of AMs on day 6 following influenza infection.

4. Recruited Mo-AMs are responsible for the inflammatory response against influenza virus.

5. Our RNA sequencing data identified several metabolic genes including *Irg1, Hk2, Pfkfb3 and Ca2* as top most upregulated genes besides antiviral response genes. Influenza virus induces glycolytic genes and glycolysis in macrophages as well as Irg1, which encodes a mitochondrial enzyme that catalyzes the conversion of aconitate to itaconate. Influenza A-induced metabolic changes are required to drive inflammatory response against influenza virus.

6. β_2AR signaling in macrophages may regulate influenza-induced metabolic changes.

3b4. Other achievements

Nothing to report.

3c. What opportunities for training and professional development has the project provided?

We actively participate in the training of physician-scientists and PhD scientists by providing them with the environment, tools and training necessary for their success and encourage them to pursue a career in environmental health sciences. The project has provided direct training to one MD post-doctoral fellows (Recep Nigdelioglu, MD) and one PhD post-doctoral fellow (Parker Woods, PhD) who both worked on acute lung injury and specifically on the influenza A-induced acute lung injury. The project also provided training to another MD post-doctoral fellow (David Wu, MD, PhD). While Dr. Wu did not directly work on the project, he developed new laboratory skills as well as training in bioinformatics during his time in the lab. All trainees had the opportunity to attend local and national conferences to improve their knowledge and present their work. I have provided formal mentorship to all of them.

Dr. Nigdelioglu published two first author manuscripts, one in *JBC* and another one in the *American Journal of Respiratory Cell and Molecular Biology* in the last 12 months. He completed his American Heart Association post-doctoral fellowship award in June 2017. He is currently a resident in pathology at Loyola University. He will pursue a research oriented career on their physician scientist track.

Dr. Parker's research focus is influenza A virus and influenza-induced acute lung injury. He led the efforts to establish the PKH26 dye based evaluation of different populations of of will pursue a MS degree in bioinformatics. He submitted an F32 to study the mechanisms by which macrophage cellular metabolism contributes to influenza A virus-induced acute lung injury. He will also submit an American Heart Association post-doctoral fellowship award application in November.

While Dr. Wu did not directly work on this project he has gained experience in influenza A model of acute lung injury, molecular biology techniques (e.g., qRT-PCR, western blot, ELISA, RNA-seq) that we employ in our laboratory. Furthermore, he has received training in bioinformatics and gene ontology analysis/IPA. He has recently published a first author paper in *Elife* and short review in *Athero Thromb Vasc Biol*. He also had a first co-author paper in the *American Journal of Respiratory Critical Care Medicine*. This manuscript investigated the role of KLF2 in different models of acute lung injury including influenza A virus. Dr. Wu received an F32 Kirschstein-NRSA Individual Fellowship application under my mentorship in 2016. He has plans to submit a K08 award under my mentorship in February 2018.

3d. How were the results disseminated to communities of interest?
Nothing to report.

3e. What do you plan to do during the next reporting period to accomplish the goals?
Aim 1. We anticipate that we will complete the experiments that we proposed in Aim 1.2., and remaining analysis that we proposed in Aim 1 in the next 3 months (by the end of month 15). We will also complete the RNA sequencing once samples from experiments in Aim 1 are completed.

Aim 2. Given that we have resolved all major issues that we encountered in year 1, we do not foresee any issues completing the experiments in Aim 2 including the tasks to meet the milestones as proposed.

4. IMPACT
4a. What was the impact on the development of the principal discipline(s) of the project?
We discovered that recruited monocyte-derived macrophages play a critical role in the development of acute lung injury. We also found that the β_2-agonists mediate their effects via β_2AR signaling on monocyte-derived macrophages. These findings suggest that any intervention focusing on macrophages should target primarily monocytes before they are recruited to lungs. Furthermore, we discovered that β_2AR signaling may mediate its effects on proinflammatory response against influenza via metabolic reprogramming, which is upstream of the inflammatory response. These discoveries have the potential to change our approach in finding new therapies for influenza infection as well as acute lung injury.

In addition to the new knowledge about the mechanisms of acute lung injury and the role of β_2AR signaling, we developed and validated the dye-based method to distinguish the lung resident macrophages from those that are recruited from the blood. This approach simplifies the assessment of the role of different subpopulations of macrophages to lung injury, eliminates the need for bone marrow chimera generation and its potential limitations on findings resulting from immunosuppression.

4b. What was the impact on other disciplines?
Nothing to report

4c. What was the impact on technology transfer?
Nothing to report

4d. What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS

5a. Changes in approach and reasons for change

There have been no changes in the objectives or the scope of the project. The specific aims that we proposed remain the same.

5b. Actual or anticipated problems or delays and actions or plans to resolve them

We had an anticipated delay in starting the chimera experiments because initial experiments using our $\beta_2AR^{-/-}$ and C57BL/6 ($\beta_2AR^{+/+}$) mice led to immune response consistent with rejection. We determined that our $\beta_2AR^{-/-}$ mice was not fully backcrossed to C57BL/6. With the help of speed congenic system, we fully-backcrossed $\beta_2AR^{-/-}$ mice to C57BL/6 after two additional crosses. Although this process led to an approximately 6 months of delay in starting the chimera experiments, it is now resolved. We expect to complete all experiments that were delayed in the next 3 months.

5c. Changes that had a significant impact on expenditures

Because of the delay starting in chimera experiments as explained above, we could not complete all experiments in Aim 1.2 as well as the RNA sequencing in Aim 1. As we proposed, the RNA sequencing will be postponed until all samples in Aim 1 are collected to minimize batch effect on the results.

5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

5d1. Significant changes in use or care of human subjects.

Not applicable. There are no human subjects in this application.

5d2. Significant changes in use or care of vertebrate animals.

None.

5d3. Significant changes in use of biohazards and/or select agents

None.

6. PRODUCTS

Publications, conference papers, and presentations

We have not had any products (publications or presentations) during the first year of the grant. We plan to submit 1-2 abstracts to the American Thoracic Society for consideration for presentation during the ATS International Conference in May 2018. We also plan to submit two manuscripts in the next year; one will be on the role of tissue resident and monocyte derived macrophages on influenza-induced acute lung injury and the second one will on the role of cellular metabolism (glycolysis and Irg1) in β_2AR signaling in macrophages as suggested by our recent findings.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7a. What individuals have worked on the project?

Name	Gokhan M. Mutlu, MD
Project Role	PI
Researcher Identifier (ORCID ID)	0000-0002-2056-612X
Nearest person month worked	12
Contribution to Project	Responsible for overall oversight of the project including designing, planning and supervision of experiments and interpretation of the data.

Funding Support	

Name	Anne Sperling, PhD
Project Role	Co-I
Researcher Identifier (ORCID ID)	0000-0002-4265-9212
Nearest person month worked	12
Contribution to Project	Assisted with flow cytometry experiments.
Funding Support	

Name	Jorge Andrade, PhD
Project Role	Co-I
Researcher Identifier (ORCID ID)	0000-0002-1261-6588
Nearest person month worked	12
Contribution to Project	Assisted with bioinformatics
Funding Support	

Name	Recep Nigdelioglu
Project Role	Post-doctoral fellow
Researcher Identifier (ORCID ID)	
Nearest person month worked	6
Contribution to Project	Performed experiments (RNA isolation, qRT-PCR, ELISA)
Funding Support	AHA 15POST255900003

Name	Kaitlyn Sun
Project Role	Lab technician
Researcher Identifier (ORCID ID)	
Nearest person month worked	3
Contribution to Project	Performed bone marrow chimeras, influenza A model of ALI. Assisted in assessment of cellular metabolism.
Funding Support	

Name	Angelo Meliton, MD
Project Role	Lab technician
Researcher Identifier (ORCID ID)	
Nearest person month worked	3
Contribution to Project	Performed bone marrow chimeras, influenza A model of ALI. Performed crossing and breeding of mice.
Funding Support	

Name	Parker S. Woods, PhD
Project Role	Post-doc
Researcher Identifier (ORCID ID)	
Nearest person month worked	12
Contribution to Project	Performed experiments, optimized influenza A-induced ALI model, developed PKH26 methodology. Performed crossing and breeding of mice.
Funding Support	T32 HL007605

7b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Dr. Mutlu's R21ES25644 grant has ended in April 2017 and R01ES015024 grant is on no cost extension.

7c. What other organizations were involved as partners?
Not applicable. Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable. Nothing to report.

9. APPENDICES
None.